

In re: Vitek
Serial No.: 09/744,133
Filed: 20 March 2001
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Remarks

This is in response to the Official Action of November 13, 2003. The points raised therein are addressed below in the order originally set forth.

Claim 1 is amended to better define "human pattern of expression". Support for this amendment is found in the specification at page 3, lines 16-21.

New claim 11 (support for which is found at page 3, lines 23), further characterizes "a human pattern of expression" and is added to complete the record.

A Rule 132 Declaration of Michael P. Vitek demonstrating mice of the invention is submitted concurrently herewith.

Claims 1-10 stand rejected under the first paragraph of 35 USC 112 as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor had possession of the claimed invention at the time the application was filed. This rejection is traversed for the same reasons set forth in applicants previous response, in view of the evidence set forth in the Rule 132 declaration submitted concurrently herewith, and in light of the further amendments clarifying the claims as set forth above.

In the most recent official action it is said that applicants have not described the characteristics of the mouse and how it be distinguished from other mice. As noted above, such description is provided at page 3 of the specification, and claim 1 has been amended to provide a simple means for distinguishing mice of the invention from other mice. Claim 11 provides a further means for distinguishing and is added to complete the record. For these reasons, it is respectfully submitted that this rejection should be withdrawn.

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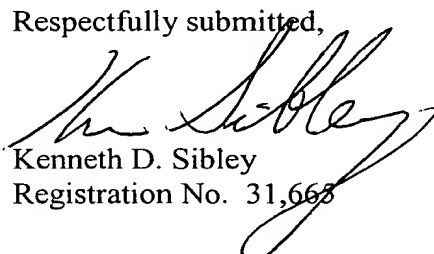
Claims 1-10 stand rejected under 35 USC 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. This rejection is respectfully traversed for the reasons set forth above, and for the reasons set forth in applicants previous response.

Claims 1-10 stand rejected as indefinite under 35 USC 112. This rejection is respectfully traversed for the reasons set forth above, and for the reasons set forth in applicant's previous response.

Claims 1-2 stand rejected as obvious under 35 USC 103 over Woi et al. in view of Chartrain et al. and Cameron. This rejection is respectfully traversed for the reasons set forth in applicant's previous response, and because the references do not teach the combination of features including the further recitation of characteristics set forth in claim 1.

It is submitted that this application is in condition for allowance, which action is respectfully requested.

Respectfully submitted,


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Enclosure: Rule 132 Declaration of Michael P. Vitek

373362



Attorney Docket No. 5405.214

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Michael P. Vitek

Group Art Unit: 1632

Serial No.: 09/744,133

Examiner: Ram R. Shukla

Filed: 20 March 2001

For: **TRANSGENIC MODEL OF HUMAN OXIDATIVE STRESS**

April 14, 2004

Commissioner for Patents
Post Office Box 1450
Alexandria, VA 22313-1450

Declaration of Michael P. Vitek, Ph.D.**pursuant to 37 C.F.R. § 1.132**

I, Michael P. Vitek, Ph.D., do hereby declare and state as follows:

1. I am an Associate Research Professor in the Division of Neurology, Department of Medicine with the Joseph and Kathleen Bryan Alzheimer's Disease Research Center at the Duke University Medical Center. My research interests include identifying the underlying causes of neurodegenerative diseases, such as Alzheimer's disease. At present, my community service includes participation on the Alzheimer's Association Medical and Scientific Advisory Board and on the Neurological Sciences III Study Section for the National Institutes of Health extramural research program. I have previously served in a similar capacity for the American Health Assistance Foundation and the Long Island Alzheimer's Foundation. I have also had the pleasure to serve as a scientific consultant for various biotechnology companies.
2. I am the named inventor on the above-referenced application, U.S. Application Serial Number 09/744,133 (*hereinafter*, "the '133 Application").
3. This Declaration is submitted to attest to the fact that a transgenic mouse that expresses human inducible nitric oxide synthase (iNOS) as outlined in Examples 1-3 in the '133 Application and concomitantly, a mouse that displays human patterns of nitric oxide production, has been constructed in my laboratory.

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4. The iNOS protein is encoded by the NOS2 gene and is found in both mouse and humans. Although the function of iNOS is similar in both mouse and in humans, the regulation of iNOS expression differs significantly between the species. The most striking differences between the mouse and human NOS2 genes are located in the promoter regions, where the number, type and location of transcription factor binding sites differ. These differences in regulatory regions indicate that the mouse and the human NOS2 gene require different types and numbers of factors (patterns) for stimulation of gene expression.
5. As outlined in the attached paper (Exhibit A) and shown in Figures 1A and 1B of Exhibit A, my laboratory has cloned the human NOS2 gene, and introduced this gene into iNOS knockout mice, i.e., mice that do not express the murine NOS2 gene, thus producing a humanized NOS2 mouse, i.e., a mouse that expresses only human iNOS gene products and no murine iNOS gene products.
6. The resulting humanized NOS2 transgenic mouse also possesses the human NOS2 regulatory components upstream from the iNOS coding region. Nitrite production as an indirect measure of NO release was examined in brain tissue and murine peritoneal macrophages from humanized NOS2 transgenic mice in response to murine and human patterns of immune stimulation and compared with wild-type control mice that express only murine NOS2.
7. Murine peritoneal macrophages from humanized NOS2 transgenic mice were stimulated with IL-1b and IFNg, typical to human patterns of immune stimulation, and assayed for nitrite production (see Figure 3, Exhibit A). Nitrite production in response to human patterns of immune stimulation was observed in the peritoneal macrophages from the humanized NOS2 transgenic mouse. Nitrite production is not observed in normal wild-type mouse peritoneal macrophages stimulated with IL-1b and IFNg.
8. The results presented in Exhibit A indicate that the humanized NOS2 transgenic mouse described in Exhibit A expresses NOS2 in response to stimulation by factors

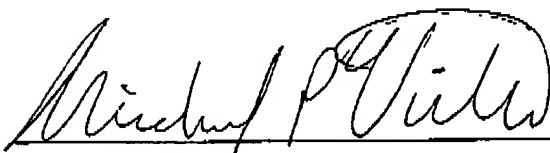
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typical in humans, and therefore provides a useful animal model for the study of NO production in response to human-specific patterns of nitrosative stress.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United State Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Michael P. Vitek, Ph.D.

12 May 04

Date

Attachments: Exhibit A

Nitric Oxide Production in a Transgenic Mouse Displays Human Patterns of Nitric Oxide Production

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EXHIBIT A
09/744,133

Abstract:

Chronic inflammation is a common component of multiple human diseases in the central nervous system and periphery. To understand how human inducible nitric oxide synthase (iNOS) contributes to chronic inflammation seen in chronic neurodegenerative pathophysiologies such as Alzheimer's disease, we constructed a transgenic mouse model that expresses only human iNOS. Using RT-PCR and Western blotting, these mice were shown to express only human iNOS RNA and protein in stimulated whole brain and peritoneal macrophages. Since microglia are the brain-specific macrophage, we studied NO responses in peritoneal macrophages as a model for brain microglia. Specifically, we measured nitrite production as an indirect measure of NO release, and iNOS catalytic activity, a direct measure of iNOS enzymatic function. Nitrite measured from stimulated peritoneal macrophages from adult (4-5 months) and aged (20-24 months) mice displayed a human pattern of NO production. These data show that this mouse is an excellent tool to study human-specific patterns of nitrosative stress that contribute to neuroinflammation in a mouse model system.

Introduction:

Inducible nitric oxide synthase (iNOS) is a member of the family of nitric oxide synthases that produce nitric oxide (NO) from the enzymatic conversion of L-arginine to L-citrulline. The iNOS protein controls several aspects of inflammation, including mediating the microbial and tumoricidal response to infection. It regulates a high output, highly regulated NO output that can be beneficial (antiinflammatory, antiapoptotic) or deleterious (), depending on the cellular microenvironment. Multiple diseases are linked to iNOS physiology and pathophysiology, including arthritis, inflammatory bowel disease, malaria, and, more recently, neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).

The iNOS protein is encoded by the NOS2 gene located on chromosomes in mouse and humans, respectively. Although the function of iNOS is similar in both mouse and humans, NOS2 gene regulation displays remarkable species specificity. The most striking differences are located in the NOS2 promoter for each species where the number, type, and location of transcription factor binding sites differ. The regulatory sequences that are necessary and sufficient for mouse NOS2 transcription are located -1.6 kb upstream of the transcription start site and contains primarily NF-kB, LPS, and interferon gamma (IRE) immune response elements (). In contrast, the NOS2 promoter

Methods:

Transgenic Mouse Construction-

The human NOS2 gene sequence was used to obtain several PAC clones of human genomic DNA containing all 65 kb of the human NOS2 gene, including 15 kb of gene regulatory sequences flanking 40 kb of exons and introns (insert ref). Each clone was subjected to multiple PCR reactions, sequenced, and restriction mapped to confirm that they were positive for and correctly matched the published promoter/exon-1 region to exon 27 region of the human iNOS gene. A single PAC clone meeting the appropriate criteria was used to generate a transgenic mouse. The purified PAC clone was purified and linearized with Not I. Not I does not cleave the human NOS2 gene (ref). Linearized DNA was injected into the pronucleus of a fertilized mouse egg at the Duke University Transgenic Mouse Facility using standard methods. Four F0 founders were identified and designated A-D. Genomic DNA was extracted from the tail of each founder and subjected to PCR using primers from the human NOS2 promoter/exon 1 region (forward primer= 5'-CCTTTCCCTTCCAAAAACCTC-3'; reverse primer= 5'-TCACCCAACCCACCTCTTTC-3'.) Genomic DNA from founders was also subjected to Southern blotting. DNA was digested with Hind III and hybridized with a pIN-2 probe, spanning positions +2401 to +4203 of the human iNOS cDNA and corresponding to exons 18-27 of the human iNOS gene. When hybridized to transgenic mouse genomic DNA, presence of the human iNOS gene is indicated by multiple bands of 3.6 kb, 4 kb, 4.5 kb, 6 kb, 8 kb, 9.5 kb, and 12 kb, while the presence of mouse iNOS gives one 7.6 kb band. Transgenic mice contained both human and mouse specific bands as described. A second complementary Southern blot was employed utilizing a BamHI digest to produce a 14 kb band in non-transgenics and 14 kb and 2 kb bands in transgenic animals using a probe corresponding to human iNOS exon 18. F₀ mice containing the human NOS2 gene were mated to wild-type (WT) C57Bl/6 mice (Jackson Labs, Bar Harbor, ME) to produce a hemizygous F₁ line used for further propagation. Lines A-C were selected for further propagation and study.

Preparation of a Humanized NOS2 mouse-

F1 progeny from lines A-C were then mated to iNOS knockout mice in two subsequent breedings (iNOS-KO) (Jackson Labs) to generate a hemizygous humanized iNOS mouse, i.e. a mouse that expresses only human iNOS gene products and no mouse iNOS gene products; these mice were designated: hNOS2Tg/mNOS2KO. The presence of the human iNOS gene was confirmed using promoter/exon 1 primer pairs described above, producing an expected 477 bp product or exon 27 primer pairs (get primer sequence) to produce a 600 bp (???) product. Animals from the B line were the most robust breeders and were used for the duration of all further studies.

Cell and Tissue Collection-

Whole mouse brains and peritoneal macrophages were collected from mice injected intraperitoneally (i.p.) with NaIO₄ (Sigma, St. Louis, MO) or vehicle (sterile 1X PBS). Briefly, iNOS induction was elicited by two intraperitoneal (i.p.) injections of NaIO₄ as previously described (Weinberg, 1983 #29; Weinberg, 1981 #514). Mice received 1 ml 5 mM NaIO₄ followed on Day 0, followed a second injection on Day 3, and were sacrificed on Day 4 of the experiment. Mice were rendered unconscious using carbon dioxide and subsequently decapitated. Whole brain tissue from female and male hiNOSTg/miNOSKO mice was collected for RNA and protein analysis; brains were separated into two hemispheres, snap frozen in liquid nitrogen, and stored at -80°C. Peritoneal macrophages were collected in 3-5 ml of ice cold 10 units/ml heparin in 1X PBS and pooled together depending on genotype and injection paradigm. Macrophages were washed using distilled water to remove red blood cells followed by addition of 2X PBS to restore isotonicity. Cells were centrifuged at 1000 rpm for 10 min; supernatant was aspirated followed by resuspension of the pellet in 15-20 ml macrophage media: 1% L-glutamine, 1% penicillin/streptomycin, 1% HEPES, 10% fetal calf serum in high glucose DMEM (all purchased from Gibco BRL, Rockville, MD.)

Treatment of Peritoneal Macrophages-

Peritoneal macrophages were plated in 96 well plates at a density of 1×10^5 cells/well in 200 µl of macrophage media and incubated at 37°C in 95% O₂/5% CO₂. To induce iNOS and NO production, cells were treated with various inflammatory stimulants including: mouse recombinant interferon γ , polyinosinic:polycytidylic acid (PIC), lipopolysaccharide (LPS): E.coli serotype 0111:B4 (all purchased from Sigma), human recombinant interferon γ , mouse recombinant IL-1 β , human recombinant IL-1 β , human TNF α (R&D Systems, city, state). Cells were treated for approximately 5 days (72-120 h) in 200 µl serum free macrophage media.

RNA Preparation and RT-PCR-

RNA was isolated from peritoneal macrophages and brain tissue using TRIzol (Gibco BRL, Rockville, MD), according to manufacturer's instructions. RNA concentrations were measured using a Beckman spectrophotometer (city, state). For RT-PCR, first strand DNA synthesis was performed using Qiagen's Sensiscript (for peritoneal macrophage RNA) or Omniscript (for whole brain RNA) Kit (city, state) according to manufacturer's directions. A 1 µl aliquot of product was used in the second strand standard PCR reaction using human iNOS specific primers exon27for 5'- -3' and exon27rev 5'- -3' with the following cycles: 94 C for 5', followed by denaturing - 1' at 94 C, annealing - sec at 60 C, extension for 1' at 72 C, for 30 cycles followed

by a final extension step for 7 min at 72 C. GAPDH primers. GAPDHF 5'- 3', and GAPDHR 5'- 3', were used as internal controls using PCR cycles previously described except that a 55 C annealing temperature was used for 25 cycles. Gels were stained using SYBR green (Molecular Probes, city, state) and visualized using a KODAK Image Station 440CCF System (Eastman Kodak, Rochester NY). Kodak 1D Image Analysis Software (Eastman Kodak, Rochester NY) was used for gel image analysis.

Western Blotting-

Peritoneal macrophages and whole brain tissue were sonicated in 1 ml or 2 ml, respectively, of protease inhibitor solution (Sigma). Protein concentrations were measured using BCA (Pierce,) or BioRad Protein Reagent (BioRad,) with BSA (ug/ml) as standard according to manufacturer's directions. 20-40 ug protein was loaded onto a 10%-20% Tricine gel (Invitrogen) in 2X Laemmli sample buffer (Novex?) and run at 100 mV for 2 hrs. Proteins were then transferred onto PVDF membrane (Millipore,) for 2hrs, blocked into Blotto (5% dry milk in TBST) at room temperature for 1 hr, followed by incubation in human iNOS primary antibody at 1:1000 (R&D) or mouse/human iNOS antibody at 1:1000 (Transduction Laboratories,), or GAPDH as a loading control (1:10000, Sigma?). Blots were washed three times in TBST followed by 1 hr incubation with the appropriate secondary antibody, subjected to 3 washes in TBST, followed by visualization using ECL-Plus chemiluminescence (Amersham,), and analyzed using a Kodak Imager and its accompanying software.

Measurement of nitrite and total protein-

Nitrite levels were determined by combining 100 µl media with 100 µl Griess reagent and incubated at room temperature for 20 minutes. Griess reagent was prepared by mixing equal volumes of reagent #1 (1% sulfanilic acid/5% phosphoric acid) and reagent #2 (0.1% naphthylethylenediamine) (all purchased from Sigma). Additional nitrite levels were also measured by injecting 50 µl media sample into a Sievers 280 NOA analyzer (Boulder, CO). Total protein (µg/well) was measured using the BCA method (Pierce, Rockford, IL) according to manufacturer's instructions with BSA (µg/ml) as standard. Both Griess and BCA values were measured using a Molecular Devices Theromax Microplate Reader (Menlo Park, CA) at OD₅₆₂. Nitrite levels were expressed as µM NO₂-/µg protein. Cell viability was measured using a standard MTT assay. Briefly, cells were incubated with 25 µl of 5 mg/ml MTT (Sigma) prepared in 1X PBS at 37°C, 95%O₂/5% CO₂ for 4 h, followed by addition of 100 µl lysis buffer overnight to solubilize the stable mitochondrial purple foramazoan product, and read at OD₅₆₂.

Measurement of iNOS Activity-

Nitric oxide synthase in elicited, immune-stimulated peritoneal macrophages was performed measuring the conversion of 14C-L-arginine to 14C-L-citrulline. Cell lysates were obtained by removing the treatment media and collected using a cell scraper into 1.5ml macrophage NOS activity buffer (ultrapure water (Gibco), 1X protease inhibitors (Sigma), 100uM PMSF). Cells were pelleted for 10 minutes at 1000rpm, 4 C; the pellet was resuspended and lysed using vigorous pipetting in 30 – 40 ul macrophage NOS activity buffer. The solution was centrifugated at 14,000rpm for 25 minutes at 4 C. Total protein content of the cell lysate was measured using the BioRad Protein Reagent (BioRad,). Activity reactions were performed in triplicate using 10ul protein lysate in a 50ul total reaction volume (). Reactions were carried out for 60 minutes at 37 C. Each reaction was

terminated by eluting the reaction mixture over a Dowex 50WX8 ion exchange resin (converted to Na⁺ form) and washing in 3 volumes of stop buffer (). Total dpm of ¹⁴C-L citrulline were counted a Beckman Tri-Carb scintillation counter (). All values were expressed as pMol/mg protein/hour.

Statistical Analysis-

Nitrite (μM)/μg protein is expressed as mean ± SEM. Each data point represents the mean of at least six wells. Data were analyzed using two-tailed paired or unpaired student's t-test and one-way or repeated measures ANOVA and Tukey's post test as appropriate with Graphpad Prism and Instat software packages (San Diego, CA). Values of p < 0.05 (*) were considered significant, p < 0.01 (**) very significant, and p < 0.001 (***) extremely significant.

Results:

Expression of hiNOS mRNA and protein-

Total RNA and protein were collected from whole brain tissue and murine peritoneal macrophages from male and female human iNOS transgenic mice to examine tissue specific expression levels of human iNOS in stimulated (5 mM NaIO₄, i.p.) and unstimulated cells. Figure 1A illustrates that a 2XX bp human iNOS fragment is expressed only in brains of male (lanes x – x) and female (x – x) human iNOS transgenic mice, while no PCR fragment was detected in wildtype C57Bl/6 mice. GAPDH fragment intensity was compared to human iNOS fragment intensity in each individual lane and no significant differences were detected in mRNA levels between males and females or between 5mM NaIO₄ or 10% peptone stimulation (data not shown). Figure 1B illustrates that a 130 kD band for human iNOS was present in stimulated whole brain tissue from both male and female mice using Western blotting, with GAPDH used as a loading control. Human iNOS fragment intensities for each sample were compared to GAPDH and found not to be significantly different between males or females (data not shown). These results demonstrate that only human iNOS mRNA and protein are expressed in this transgenic mouse.

Nitrite production in human NOS2 transgenic mice: mouse versus human-

One goal of the study was to determine if the difference in levels of nitrite production between rodents and humans was due to the presence the structure of the human or mouse genes, or due to the unique cell biology of human and mouse cell. These hypotheses were tested by measuring nitrite production using both human and mouse recombinant cytokines in combination with typical murine and human patterns of immune stimulation. Figure 2 shows nitrite production in 4-5 month old male peritoneal macrophages stimulated for 5 days using typical murine patterns of immune stimulation that included: Poly I:C (PIC), a viral mimetic; lipopolysaccharide (LPS), a bacterial endotoxin; and murine recombinant interferon gamma (mIFNγ). Nitrite was measured using the Griess reaction and chemiluminescence and found to be similar to published nitrite levels from stimulated human monocytes (insert ref). Nitrite levels were detectable at the only at the nmol level after 5 days, a result in sharp contrast to normal murine nitrite levels that are easily detectable at the umol level after 24 – 48 hrs. Stimulation using mIFNγ (2.5ng/ml) and LPS (500ng/ml) produced the highest level of nitrite output at 800 nmol/ug. All nitrite production was significantly higher than that from untreated peritoneal macrophages (p < 0.001, ANOVA).

Nitrite production from 4-5 month old male peritoneal macrophages stimulated using typical human patterns of immune stimulation is shown in Figure 3. Nitrite production in peritoneal macrophages was lower in these cells than the cells treated with mouse immune stimulation (Figure 2); the highest level of nitrite production was at 300 nmol/ug versus 800 nmol/ug for murine stimulation. Murine IL-1 β (0.2 ng/ml) and human IL-1b (0.2 and 2 ng/ml) were capable of producing a nitrite response alone which is normally not detectable in normal wildtype mouse peritoneal macrophages. Combinations of murine and human IL-1b with both mouse and human IFNg were also capable of synergistically producing an immune response that was significantly higher than treatment with IL-1b alone ($p < 0.05$ – insert statistic from raw data). Immune stimulation was also detected using human TNFa (10ng/ml) that were significantly higher than unstimulated cells. However, immune stimulation of transgenic macrophages with a combination of cytokines including IFNg, IL-1B, and TNFa produced a nitrite response that was insignificant compared to unstimulated macrophages.

Nitrite Production in Aged NOS2 Transgenic Mouse:

Human iNOS transgenic mice were aged to 20-24 months and nitrite production was measured to assess differences in the human iNOS inflammatory response during advanced age.

iNOS Activity in NOS2 Transgenic Mouse

Discussion

Multiple studies using human mononuclear phagocytes have reported nitrite production only by using a combination of various human cytokines (insert ref), while others have reported

Figure Legends

Acknowledgements: We wish to acknowledge Michael H. Herbstreith for excellent technical support for maintenance of these mouse strains and Dr. Carol A. Colton for helpful comments and insightful discussions.

References:

